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New features in the genus *Ilarvirus* revealed by the nucleotide sequence of *Fragaria chiloensis latent virus*

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Abstract

Fragaria chiloensis latent virus (FClLV), a member of the genus *Ilarvirus* was first identified in the early 1990s. Double-stranded RNA was extracted from FClLV infected plants and cloned. The complete nucleotide sequence of the virus has been elucidated. RNA 1 encodes a protein with methyltransferase and helicase enzymatic motifs while RNA 2 encodes the viral RNA dependent RNA polymerase and an ORF, that shares no homology with other *Ilarvirus* genes. RNA 3 codes for movement and coat proteins and an additional ORF, making FClLV possibly the first *Ilarvirus* encoding a third protein in RNA 3.

Phylogenetic analysis reveals that FClLV is most closely related to *Prune dwarf virus*, the type member of subgroup 4 of the *Ilarvirus* genus. FClLV is also closely related to *Alfalfa mosaic virus* (AlMV), a virus that shares many properties with ilarviruses. We propose the reclassification of AlMV as a member of the *Ilarvirus* genus instead of being a member of a distinct genus.

Keywords: *Fragaria chiloensis latent virus*; *Ilarvirus*; *Prune dwarf virus*; *Alfalfa mosaic virus*

Fragaria chiloensis latent virus (FClLV) was first identified in 1993 (Spiegel et al., 1993) and was detected only in *Fragaria chiloensis* (Chilean strawberry) plants that had been collected in Chile (Cameron et al., 1993). *F. chiloensis* is distributed along the west coast of the Americas from Alaska to southern California and from the central coast of Chile south (Daubeny, 2003). FClLV is a member of the genus *Ilarvirus* in the family *Bromoviridae* (Spiegel et al., 1993). Ilarviruses are positive-strand tripartite RNA viruses that encode four or five proteins (Bol, 1999). RNA 1 is monocistronic, encoding a protein with methyltransferase and helicase signature motifs. In the case of *Brome mosaic virus*, the type member of the family *Bromoviridae*, this molecule has been shown to be involved directly in the replication complex not only as an enzyme but also as the anchor of the complex onto cell membranes (den Boon et al., 2001). RNA 2 encodes an RNA-dependent-RNA polymerase (RdRp). Some members of the family (cucumoviruses and some ilarviruses) contain a second gene on the RNA 2 that encodes a protein that is involved in suppression of RNA interference (RNA silencing) (Brigneti et al., 1998). Movement and coat proteins (MP and CP, respectively) are encoded by RNA 3. The coat protein is expressed through a subgenomic RNA 4 and in addition to protecting the genome is required for virus movement and genome activation (Jaspars, 1999; Neeleman et al., 2004). The requirement of CP for genome activation is a property of the ilarviruses and *Alfalfa mosaic virus* (AlMV), viruses that are very similar, a property that is emphasized by the molecular data presented in this communication.

In addition to FClLV and the more than 15 viruses identified in strawberry (Spiegel and Martin, 1998; Tzanetakis and Martin, 2004; Tzanetakis et al., 2005a; Tzanetakis and Martin, unpublished data) there are another two ilarviruses infecting strawberry, Strawberry necrotic shock virus (Tzanetakis et al., 2004b) and *Apple mosaic virus*

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(Tzanetakis and Martin, 2005). The fact that strawberry plants generally are asymptomatic when infected with a single virus, while many cultivars exhibit decline symptoms when infected with multiple viruses (Converse, 1987), makes virus detection a complex undertaking in this crop. Until recently, reliable detection was based on grafting of suspicious material onto *F. virginiana* and *F. vesca* clones (Spiegel and Martin, 1998) and the symptomatology observed. In recent years, detection of strawberry viruses has improved radically with the usage of RT-PCR (Thompson et al., 2003; Tzanetakis et al., 2003, 2004a,b). Although FClLV can be detected using monoclonal and polyclonal antibodies, pollen and seed transmission, and the utilization of *F. chiloensis* in breeding programs has necessitated the need for a more sensitive detection assay. Thus, we cloned and sequenced cDNA derived from dsRNA from FClLV infected plants. A reverse transcription-polymerase chain reaction (RT-PCR) test that amplifies a fragment of RNA 2 has been developed. Phylogenetic analysis utilizing the signature motifs of RNA 1 and 2 and the MP and CP assigns the virus into subgroup 4 of the *Ilarvirus* genus along with *Prune dwarf virus* (PDV).

For all enzymatic reactions described hereafter reagents from Invitrogen Corp. (Carlsbad, CA) were utilized unless otherwise stated. For detection purposes, RNA was extracted and reverse transcribed as described previously (Tzanetakis et al., 2004a). PCR was performed using the Platinum Taq® polymerase and primers FC polF (5′ ACCACTTCACCACCAGATCG 3′) and FC polR (5′ CAAGCCAACTCACCATGACC 3′). The PCR program consisted of original denaturation for 5 min at 94 °C followed by 40 cycles of 45 s at 94 °C, annealing for 30 s at 55 °C and extension for 30 s at 68 °C. The final step included incubation of the reaction at 68 °C for 10 min. Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) was performed as described previously (Wetzel et al., 1992).

Double-stranded RNA was purified using a modified version of the Yoshikawa and Converse method (1990) from the *F. chiloensis* clone CFRA 9089 maintained at the National Clonal Germplasm Repository (Corvallis, OR) and utilized for cloning. First- and second-strand cDNA synthesis were performed either according to the method of Jelkmann et al. (1989) or the Tzanetakis et al. (2005b) method, employing dsRNA extracted from 5 g of tissue. Briefly, for the latter method, dsRNA was denatured in the presence of 20 mM methyl mercuric hydroxide and 1 μl random oligonucleotide hexamers for 30 min at room temperature. Reverse transcription was performed utilizing either Superscript III® (SSIII) or Thermoscript® (TS) reverse transcriptases. After incubation of the reaction at 50 or 60 °C (SSIII and TS, respectively) for 60 min, 200 units of SSIII, 20 units of *Eco*NI (New England Biolabs, Beverly, MA) and 2 units of RNAse H were added in the reaction which was further incubated for 60 min at 37C. The reaction products were purified with the PCR purification system® (Marligen Biosciences, Ijamsville, MD) and eluted in 50 μl of water. *Taq* polymerase (New England Biolabs, Beverly, MA) (2.5 units) and the respective buffer and 0.2 mM deoxynucleotides were added and the reaction was incubated for 30 min at 75 °C, in order to fill the restriction sites and add an deoxyadenosine overhang on 3′ end of the double-stranded cDNA. The cDNA fragments were purified as described above in 20 μl of water and 4 μl were used for cloning into the pCR 4 TOPO® vector according to manufacturer′s instructions. Polymerase chain reaction (PCR) reactions were performed on the recombinant plasmids utilizing primers M13 forward and reverse primers to identify those with the largest inserts. Plasmids were sequenced utilizing an ABI 3700 DNA sequencer (Macrogen Inc., Seoul, Korea).

The sequences acquired from shotgun cloning were compared against accessions in GenBank (Altschul et al., 1997) to identify the region of the genome that was cloned. Sequences were aligned utilizing ClustalW software (Thompson et al., 1994). Oligonucleotide primers were designed after aligning at least two cDNA clones and were used to perform RT-PCR. To determine the sequence of the 5′ termini, the Gene Racer® technology was used according to the manufacturer instructions. The sequences of the 3′ termini were determined after addition of an adenosine polymer (Sippel, 1973) to the termini and RT-PCR amplification. PCR was performed with the Platinum Taq® polymerase in a program that consisted of denaturation for 5 min at 94C followed by 40 cycles of 45 s at 94 °C, annealing for 30 s at 55 °C and extension for 1–2 min at 68 °C. The reaction terminated with incubation at 68 °C for 10 min. Amplicons were cloned into a pCR 4 TOPO® vector and sequenced as described above.

The consensus sequence presented here was determined by aligning the sequences of the clones obtained by shotgun cloning and at least four clones obtained by PCR per site. The GenBank accession numbers are NC 006566 to NC 006568 (RNA 1, -2 and -3, respectively). Phylogenetic analysis was based on maximum parsimony performed on PAUP* 4.0b v.10 (Swofford, 2001) utilizing heuristic search with ten random addition replications and the tree bisection reconnection swapping algorithm. The bootstrap analysis consisted of 100 replications utilizing the same parameters as above.

FClLV has the first six and the last 61 nucleotides (nt) of the three genomic RNAs identical. RNA 1 consists of 3431 nucleotides and is monocistronic, encoding a putative protein of 121 kDa. The first start codon is found at position 26 and although not in the best context for translation initiation (Kozak, 1986), it appears to be the initiation point since the next AUG is found at position 417, inside the predicted methyltransferase domain (Rozanov et al., 1992). The putative protein is 1083 amino acids (aa) long and is most closely related to the homologous protein of PDV, sharing 55% aa identity and 75% similarity. The methyltransferase domain is found between aa 85 and 285 (Rozanov et al., 1992). An intragenic region without any identifiable motifs spans about 500 aa, followed by the helicase domain, between residues 786 and 1035 (Candresse et al., 1990). The 3′ untranslated region (UTR) extends 153 nucleotides after the stop codon and is predicted to fold into five stem-loop structures (Zuker, 2003),

similar to the 3′ UTR of the other two genomic molecules (data not shown).

RNA 2 is 2570 nt long and encodes a putative protein of 786 aa with predicted MW of 89 kDa. The initiation codon is found at position 28 while the ORF terminates at position 2388. The conserved RNA-dependant-RNA polymerase motifs identified by Koonin (1991) are found in the C′ terminus of the protein between aa 458 and 700. This region shares a high degree of similarity with all ilarviruses and AlMV and with all but one member of the genus, sharing more than 75% aa similarity with FClLV. Some members of the family encode a protein at the 3′ end of RNA 2, that is involved in RNA silencing (Li and Ding, 2001) but no ORF is found in that position of FClLV RNA 2. An ORF at an alternative reading frame was identified (Pattern/Markov chain-based viral gene prediction at softberry.com) between nt 350 and 856. The predicted 19 kDa protein has a putative transmembrane domain between residues 6 and 25 (Pasquier et al., 1999) and shares minimal similarity (42% aa similarity over the length of the peptide) with eukaryotic receptors.

RNA 3 is 2484 nt long. The putative movement protein of the virus is encoded between nt 306 and 1250. The 35 kDa protein, the largest encoded by an *Ilarvirus*, shares 72% aa similarity with the MP of PDV. A highly charged area between aa 55 and 95 was identified with a predicted α-helix between two β-sheets (Rost, 1996), similar to location and structure to regions with RNA-binding properties identified in other ilarviruses (Herranz and Pallas, 2004).

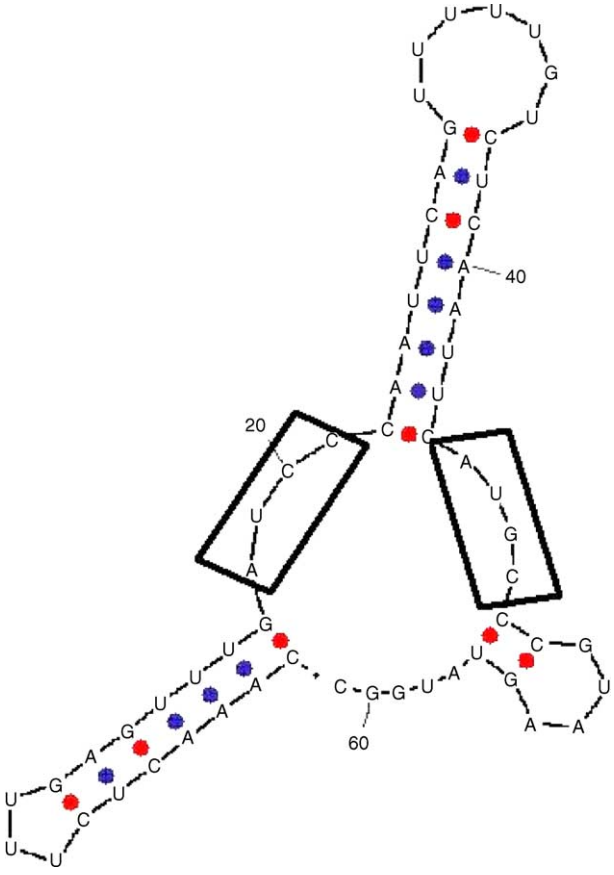
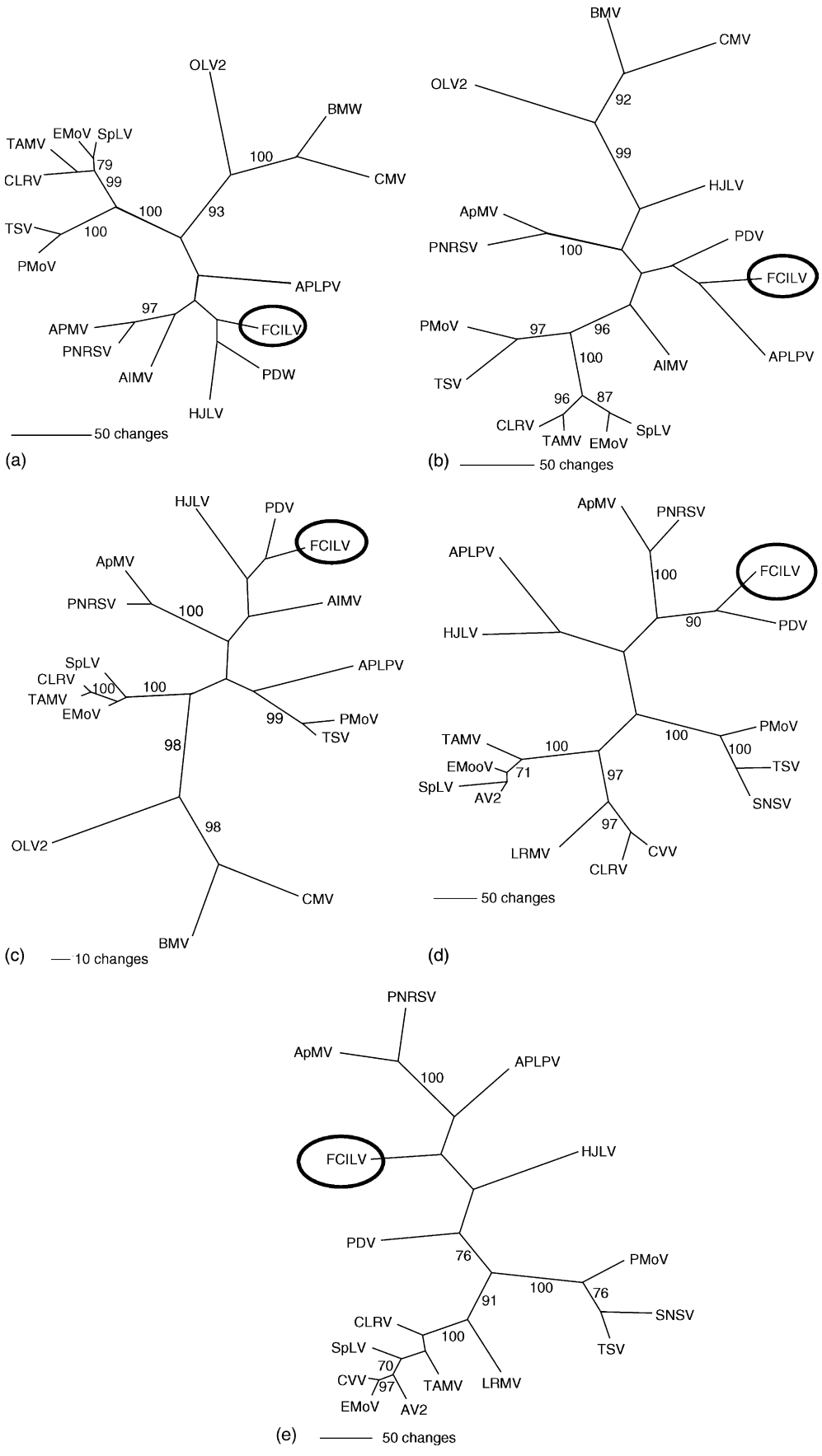


Fig. 1. Predicted folding of the last 61 identical nucleotides of the 3′ untranslated region of the genomic RNA of *Fragaria chiloensis latent virus*. The boxed sequences indicate the putative binding region of the coat protein for genome activation.

Fig. 2. (A) Phylogenetic analysis of ilarviruses and the type members of the *Bromoviridae* genera utilizing the methyltransferase conserved motifs. Abbreviations and GenBank accession numbers: *Fragaria chiloensis latent virus*, FClLV; *Prune dwarf virus*, PDV AAB 39537; *Prunus necrotic ringspot virus*, PNRSV NP 733823; *Apple mosaic virus*, ApMV NP 604469; *American plum line pattern virus*, APLPV NP 602312; *Tobacco streak virus*, TSV NP 620772; *Parietaria mottle virus*, PMoV YP 006446; *Tulare apple mosaic virus*, TAMV NP 620753; *Spinach latent virus*, SpLV NP 620677; *Elm mottle virus*, EMoV NP 619576; *Humulus japonicus latent virus*, HJLV AAS 86438; *Citrus leaf rugose virus*, CLRV NP 613282; *Brome mosaic virus*, BMV NP 041196; *Alfalfa mosaic virus*, AlMV NP 041192; *Cucumber mosaic virus*, CMV NP 049323; *Olive latent virus* 2, OLV 2 NP 620042. (B) Phylogenetic analysis of ilarviruses and the type members of the *Bromoviridae* genera utilizing the helicase conserved motifs. Abbreviations and GenBank accession numbers: *Fragaria chiloensis latent virus*, FClLV; *Prune dwarf virus*, PDV AAB 39537; *Prunus necrotic ringspot virus*, NRSV NP 733823; *Apple mosaic virus*, ApMV NP 604469; *American plum line pattern virus*, APLPV NP 602312; *Tobacco streak virus*, TSV NP 620772; *Parietaria mottle virus*, PMoV YP 006446; *Tulare apple mosaic virus*, TAMV NP 620753; *Spinach latent virus*, SpLV NP 620677; *Elm mottle virus*, EMoV NP 619576; *Humulus japonicus latent virus*, HJLV AAS 86438; *Citrus leaf rugose virus*, CLRV NP 613282; *Brome mosaic virus*, BMV NP 041196; *Alfalfa mosaic virus*, AlMV NP 041192; *Cucumber mosaic virus*, CMV NP 049323; *Olive latent virus* 2, OLV 2 NP 620042. (C) Phylogenetic analysis of ilarviruses and the type members of the *Bromoviridae* genera utilizing the polymerase conserved motifs. Abbreviations and GenBank accession numbers: *Fragaria chiloensis latent virus*, FClLV; *Prune dwarf virus*, PDV AAK 69444; *Prunus necrotic ringspot virus*, PNRSV NP 733824; *Apple mosaic virus*, ApMV NP 604470; *American plum line pattern virus*, APLPV NP 602313; *Tobacco streak virus*, TSV NP 620768; *Parietaria mottle virus*, PMoV YP 006447; *Tulare apple mosaic virus*, TAMV NP 620754; *Spinach latent virus*, SpLV NP 620678; *Elm mottle virus*, EMoV NP619575; *Humulus japonicus latent virus*, HJLV AAS 86439; *Citrus leaf rugose virus*, CLRV NP 613281; *Brome mosaic virus*, BMV NP 041197; *Alfalfa mosaic virus*, ALMV NP 041193; *Cucumber mosaic virus*, NP 049324; *Olive latent virus 2*, OLV2 NP 620043. (D) Phylogenetic analysis of the movement proteins of ilarviruses. Abbreviations and GenBank accession numbers: *Fragaria chiloensis latent virus*, FClLV; *Prune dwarf virus*, PDV AAA 46818; *Prunus necrotic ringspot virus*, PNRSV NP 733825; *Apple mosaic virus*, ApMV NP 604484; *American plum line pattern virus*, APLPV NP 602314; *Strawberry necrotic shock virus*, SNSV AAQ76575; *Tobacco streak virus*, TSV NP620773; *Parietaria mottle virus*, PMoV YP 006463; *Asparagus virus 2*, AV2 CAA 60124; *Tulare apple mosaic virus*, TAMV NP 620756; *Spinach latent virus*, SpLV NP 620680; *Elm mottle virus*, EMoV NP 619577; *Citrus variegation virus*, CVV AAC 54055; *Lilac ring mottle virus*, LRMV AAA 64839; *Humulus japonicus latent virus*, HJLV AAS 86440; *Citrus leaf rugose virus*, CLRV NP 613279. (E) Phylogenetic analysis of the coat proteins of ilarviruses. Abbreviations and GenBank accession numbers: *Fragaria chiloensis latent virus*, FClLV; *Prune dwarf virus*, PDV AF208740; *Prunus necrotic ringspot virus*, PNRSV AY037790; *Apple mosaic virus*, ApMV S78319; *American plum line pattern virus*, APLPV NC 003453; Strawberry necrotic shock virus, SNSV AY 363228; *Tobacco streak virus*, TSV NC 003845; *Parietaria mottle virus*, PMoV U 35145; *Asparagus virus 2*, AV 2 X 86352; *Tulare apple mosaic virus*, TAMV NC 003835; *Spinach latent virus*, SpLV NP 620681; *Elm mottle virus*, EMoV NC 003570; *Citrus variegation virus*, CVV AF 434911; *Lilac ring mottle virus*, LRMV U 17391; *Humulus japonicus latent virus*, HJLV AAS 86441; *Citrus leaf rugose virus*, CLRV NP 613280; *Citrus leaf rugose virus*, CLRV NP 613280.


OLV2
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TAMV
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SpLV
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SNSV
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LRMV
CVV
CLRV
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PNRSV
ApMV
APLPV
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FCILV
HJLV
PDV
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PMoV
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SNSV
CLRV
100
TSV
SpLV
70
CVV
97
TAMV
LRMV
EMoV
AV2
(e)
50 changes

The CP ORF starts at nt 1316 and terminates at nt 1975 encoding a protein of 25 kDa. The protein shows greater similarity with PDV CP (56% aa similarity) than any other *Ilarvirus*. The protein contains the conserved residues found in CP proteins of members of the genus *Ilarvirus* and AlMV involved in binding of the protein to the structures of the 3′ UTR between residues 21 and 30 (Ansel-McKinney et al., 1996). A predicted ORF of 78 aa with a mass of 9 kDa is found at the 3′ terminus of the molecule between nt 2075 and 2311. This putative peptide does not share significant similarity with any other proteins in the database.

The 3′ UTR sequences of the three RNAs of the virus share 76% nt identity. The last 61 nt, that are identical in all three molecules, are predicted (Zuker, 2003) to fold into stem-loop structures separated by AUGC spacers (Fig. 1). These structural elements have been identified in other ilarviruses and AlMV to be involved in CP binding and genome activation (Ansel-McKinney et al., 1996; Bol, 1999).

The initial study of the virus (Spiegel et al., 1993) trapped virus particles on electron microscope grids as a means to identify the relationship of FClLV to other ilarviruses. Applying this technique, the virus was found to be related to *Lilac ring mottle virus* and *Asparagus virus 2*, viruses belonging to *Ilarvirus* subgroups 7 and 2, respectively. Phylogenetic analysis of the enzymatic signature motifs of RNAs 1 and 2 and the MP and CP, revealed FCILV to be most closely related to PDV, AlMV, *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV) (Fig. 2). The close relationship of FClLV with the above mentioned viruses in combination with the recent discovery of ApMV naturally infecting strawberry (Tzanetakis and Martin, 2005), makes it possible that there are more ilarviruses infecting the crop.

In order to verify that the virus characterized was FClLV, IC-RT-PCR was utilized. Polystyrene plates (NUNC, Rochester, NY) were coated with plant sap, anti-FClLV, PNRSV or ApMV antibodies. Amplicons were acquired only from plates that were coated with the anti-FClLV antibodies (Fig. 3). The direct coating with the plant sap may have failed to give any amplicons with IC-RT-PCR due to the instability of the virions upon attachment to the matrix of the plate (data not shown). The results of IC-RT-PCR verified that the virus presented in this communication is FClLV.

The phylogenetic analysis also revealed a close relationship between FClLV and AlMV, the type member of the genus *Alfamovirus*. The data obtained in this and previous studies (Rampitsch and Eastwell, 1997; Shiel and Berger, 2000) indicate that AlMV is related more closely to some members of the *Ilarvirus* genus than are some members of the genus to each other. The only property of AlMV that sets it apart from the ilarviruses is its aphid transmissibility and this may not justify its status as a separate genus. Therefore, we would suggest that AlMV be classified as an *Ilarvirus* rather than a member of a separate genus.

The knowledge of the nucleotide sequence of FClLV allowed the development of oligonucleotide primers that can be utilized for molecular detection of the virus. The complete nucleotide sequence of FClLV revealed further complexity in the genus *Ilarvirus*, with an additional two putative ORFs not identified in other members of the group. Based on phylogenetic analysis, FClLV should be considered to be a member of subgroup 4 of the genus together with PDV, unlike the previous report based on a serological assay that showed a relationship between FCILV and *Lilac ring mottle* and *Asparagus virus 2*. This is an indication that serological relationships are not always reliable for assigning viruses into groups since some epitopes result from secondary and tertiary protein structures and it is possible that an epitope of FClLV is recognized by *Lilac ring mottle virus* and *Asparagus virus 2* antisera which resulted in the previous results.

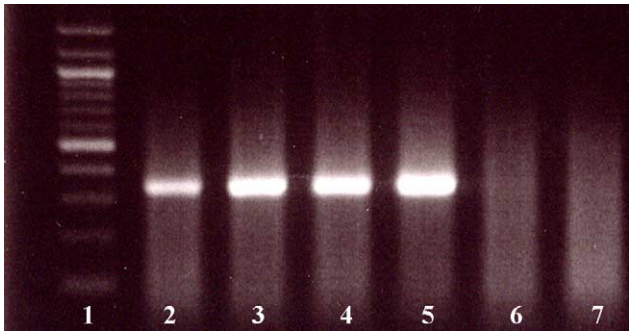

Fig. 3. Immunocapture-reverse transcription-polymerase chain reaction for verification that the virus studied was *Fragaria chiloensis latent virus*. Lane 1: 100 base-pair DNA marker (NEB, Beverly, MA); lanes 2–5: anti-*Fragaria chiloensis latent virus* antibody coated wells, National Clonal Germplasm Repository (NCGR) *Fragaria chiloensis* accession numbers CFRA 9087–CFRA 9090; lane 6: anti-*Prunus necrotic ringspot virus* antibody coated wells, NCGR *F. chiloensis* accession number CFRA 9089; lane 7: anti-*Apple mosaic virus* antibody coated wells, NCGR *F. chiloensis* accession number CFRA 9089.

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